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14. ABSTRACT

ERβ knock-out mice developed prostatic hyperplasia at late age, suggesting an important role of ERβ in the development of the prostate as well as prostate cancer. Here we describe a study that thoroughly investigates the genomic function of ERβ. A FLAG-tagged ERβ was stably expressed in MCF7 C4-12 cells, which allowed ERβ transcriptional activity to be studied in an ERα-independent background. Interestingly, in our chromatin immunoprecipitation followed by sequencing (ChIP-seq) analyses, looking at ERβ global binding sites, while the most prevalent binding motif was the canonical ERE, ~30% of ERβ binding regions also carried the binding motif of EBF1 (Early B-cell Factor 1). Further investigations revealed EBF1 down-regulated ERβ protein stability and transcriptional activity via a direct interaction. These results, at least to our knowledge, are the first to indicate crosstalk between EBF and ER activities on a large scale. Moreover, in conjunction with global run-on followed by sequencing (GRO-seq), looking at nascent RNA generated at the time of ERβ binding events, still to be completed, our results should reveal a global picture of how ERβ regulates its direct target genes, as well as the role of EBF proteins in this process.

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Genome-wide mapping; estrogen receptor beta

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INTRODUCTION

Beside being the major target of androgens, the prostate is also influenced by estrogens. In the adult male, 17-beta-estradiol (E2), classically considered the female sex hormone, is mainly produced by adipose tissue, adrenal glands, testicles and the prostate[1]. On the cellular and molecular level, E2 mainly exerts its effect via the two estrogen receptors, ERalpha and ERbeta. ERalpha, as a marker for breast cancer, has been heavily studied, leading to a myriad of improvement in breast cancer treatment and prevention[2]. On the other hand, the functions of ERbeta, the recently-discovered subtype, remain largely elusive if not controversial. Interestingly, ERbeta knock-out mice were reported to develop prostatic hyperplasia at late age[3]. Moreover, different lines of studies have suggested that ERbeta played an important role in anti-proliferation[4], immunoprotection[5], and detoxification in the prostate[6]. Therefore, ERbeta strikes as an important component in prostatic normal development as well as tumorigenesis. Because ERbeta regulates gene expression mainly at the transcription level, the proposed study is designed to map ERbeta genome-wide binding sites in ERalpha-negative cell model using ChIP-seq technology. In conjunction with nascent RNA generated at the time of binding events, detected by GRO-seq technology, our results will provide a better knowledge of ERbeta target genes as well as how ERbeta regulates their expressions on the global scale. Ultimately, this will give rise to a better model to study this nuclear receptor.

BODY

Aim1 – To identify genome-wide ERbeta binding regions

The lack of a reliable antibody for ERbeta has led to many controversies surrounding the presence and functions of this steroid receptor[8]. Due to the same reason, detecting ERbeta binding sites, via chromatin immunoprecipitation (ChIP) assays, has also been delayed. Therefore, we propose to generate cancer cell lines stably expressing ERbeta as a FLAG-tagged fusion protein (FLAG-ERbeta). Since specific and reliable antibodies raised against the FLAG are broadly available, the fusion protein will be efficiently immunoprecipitated in ChIP conditions.

In order to map ERbeta genome-wide binding sites in a non-biased manner, we choose ChIP followed by massive parallel sequencing (ChIP-seq) as our main approach. This is because, in the last couple years, this technology has been widely proven to produce reliable data with higher resolution compared to the conventional ChIP-chip assay (ChIP followed by tiling microarray).

Also, in order to study ERbeta genomic functions without the interference of ERalpha, we wished to introduce the FLAG-ERbeta into two well-characterized prostate cancer cell lines LNCaP, PC3 (in which ERalpha is undetectable), and a breast cancer cell line MCF7-C4-12 (a derivative of the MCF7 cell line which does not express ERalpha).

C4-12-ERbeta cells stably expressed FLAG-tagged ERbeta:

Due to a variety of reasons (low viral infection efficiency, low cell growth rates, etc.), we were not able to generate the prostate cancer cells stably expressing FLAG-ERbeta at first. However, the DNA construct of the fusion protein appeared to be integrated into the genome the MCF7-C4-12 cells at a much higher rate. The efficiency of viral infection could be ranked highest in MCF7-C4-12 cells, low in PC3 cells, and very low in LNCaP cells. Moreover, the MCF7-C4-12 cells grew at a very fast pace, which allowed generating material for the downstream assays much more efficiently. Therefore, we used the MCF7-C4-12 cells stably expressing FLAG-ERbeta (C4-12-ERbeta) to optimize the condition for the ChIP-seq assay, while developing the prostate cancer cells stably expressing FLAG-ERbeta.

Although we were able to express FLAG-ERbeta in LNCaP and PC3 cells, the stable expression level of the ectopic protein was too low. Moreover, these cell lines appeared to quickly lose FLAG-ERbeta expression. Meanwhile, we were advancing our analyses of ERbeta genomic functions using the C4-12-ERbeta cells. Although this cell line derived from the breast cancer cell line MCF7, the cells had gone through significant changes so that they should no longer be considered a cell model for breast cancer. However, these cells still provided sufficient cellular environment to study the biology of ERbeta.

Therefore, we decided to carry on our project using the C4-12-ERbeta as our cell model.

Mapping ERbeta genomic binding sites

C4-12-ERbeta cells were serum-starved for 24 hours before treated for 1 hour with 10nM estradiol (E2). Cells treated with the same volume of vehicle (ethanol) were used as control. The cells were crosslinked and proceeded through ChIP assay, in which anti-FLAG M2 antibody (Sigma-Aldrich) was used to pull down the FLAG-ERbeta. The immunoprecipitated DNA libraries were prepared according to the Illumina ChIP-seq library prep kit. Samples were then submitted to high throughput sequencing with Solexa technology at the IGSB High-throughput Genome Analysis Core (Argonne National Laboratory, IL).

As we proceeded through the ChIP-seq assay with the C4-12-ERbeta cells, our data confirmed the high efficiency of the optimized ChIP-seq conditions. We were also able to identify more than 5000 binding sites ERbeta binding sites in this breast cancer cell model at low stringency (5% FDR) and more than 3000 binding sites at high stringency (1% FDR). The global distribution of ERbeta binding sites at high stringency is presented in figure 1A. Moreover, our motif analysis identified binding motifs of ERs, known ERbeta interactors as well as those of novel interactors (figure 1B).

To our surprise, the binding motif of EBF1 (Early B-cell Factor 1) was highly enriched in ERbeta binding regions. Moreover, EBF1 mapping experiments revealed binding motifs of the estrogen receptors (Triggs, J., unpublished data). These evidence, at least to our knowledge, were the first to suggest a genomic interplay between EBF1 and ERbeta. Furthermore, EBF1 was downregulated in prostate cancer cells (Oncomine). This prompted us to perform downstream assays, described below, to further investigate such crosstalk.

EBF1 downregulated ERbeta transcriptional activity

We performed a luciferase reporter assay to assess if EBF1 affected ERbeta transcriptional activity. Briefly, C4-12 cells were transiently transfected with a plasmid carrying a Firefly Luciferase gene preceded by three tandem estrogen response element (ERE), a Renilla Luciferase plasmid (to serve as transfection efficiency control), and an ERbeta plasmid. These cells were also transiently transfected with a pcDNA as vector control or an EBF1 plasmid. Upon estradiol induction, ERbeta was recruited to the ERE and facilitate the expression of lucierase protein, resulting in an increase of luciferase activity. However, in the presence of EBF1, this activity was significantly reduced in a ligand independent manner (Figure 2A).

Furthermore, EBF1 also downregulated transcriptional activities exerted by ERalpha and the two chimeric receptors in a ligand independent manner. Interestingly, constructs carrying ERbeta AF1 domain (full length ERbeta and ERbeta/alpha) were more affected by EBF1 comparing to those carrying ERalpha

AF1 domain (full length ERalpha and ERalpha/beta) (Figure 2B). Such results suggested an interaction between EBF1 and the N-terminal regions of the estrogen receptors.

EBF1 inversely correlated with ERbeta protein stability

Since previous reports had been suggesting the role of the AF1 domain in the degradation of the estrogen receptors, we were interested to see if EBF1 participated in such regulation. C4-12 were transiently transfected with ERbeta in the presence or absence of EBF1. After cells had been serum starved for 24hours, cell lysates were collected after 3 hours of treatment with estradiol (E2) or vehicle. Indeed, the presence of EBF1 inversely correlated with ERbeta protein stability while leaving ERbeta transcript level unchanged (data not shown) in a ligand independent manner (figure 2C). These results further suggested the role of EBF1 in the regulation of ERbeta on the protein levels. This also explained our multiple failed attempts to co-immunoprecipitate EBF1 with ERbeta.

EBF1 directly interacted with ERbeta

Although we wished to test if EBF1 directly interacted with ERbeta, the decreased ERbeta protein level prevented such assay. In order to go about this issue, cell lysates were collected after 3hours of treatment with E2 or vehicle, in the presence of MG132, a proteasome inhibitor. In such condition, EBF1 was co-immunoprecipitated with ERbeta (Figure 2D), indicating a direct interaction.

In progress

Since our results suggested that EBF1 interacted with ERbeta somewhere in the N-terminal region of the receptor, we would like to map the particular domain(s) on ERbeta that are involved in such interaction. In order to address this question, we will use purified GST-tagged EBF1 as bait to pull down different ERbeta constructs, each carrying different ERbeta domains.

Aim 2 – To identify genes directly regulated by ERbeta

Because emerging evidence had shown that trimethylation of lysine 4 on histone 3 (H3K4me3) was a reliable marker for active promoters, we first attempted to use this as the marker for actively transcribed genes with ERbeta binding events, hence ERbeta direct target genes. However, our analyses revealed that the hormone treatment time of 1 hour was too short for differential modifications to occur.

Alternatively, recent advances in high throughput sequencing technology have allowed the development of a new technology, namely global nuclear run-on followed by sequencing (GRO-seq). This technology provides the detection of nascent RNA on a global scale[9,10]. We have successfully applied this new technology to identify ERbeta direct target genes.

GRO-seq

In order to capture gene regulation by ERbeta binding onto the chromatin, cell nuclei were extracted and collected after 1 hour of E2 or vehicle treatment (the same time point of ERbeta genomic binding events). Applying the GRO-seq technology, nascent RNA from these nuclei, which represented genes being actively transcribed, were extracted and processed for high throughput sequencing. The results were lists of genes being actively up- or down-regulated at the time of ERbeta binding events. These gene lists were overlapped with the list of genes harboring ERbeta binding sites, revealing ERbeta direct target genes. Among these direct targets, 784 genes were up-regulated, 360 of which carried the EBF1 binding motif. 480 direct targets were down-regulated, 234 of which carried the EBF1 binding motif.

In progress

In order to generate statistical powers with our GRO-seq data, biological replicates are required. These samples are being generated and will be submitted for sequencing shortly.

With the available gene list (without statistics values), we are in the process of analyzing the gene ontology for these gene lists (up- and down-regulated genes). Validating ERbeta direct target genes by using RT-qPCR is also in progress. We also wish to identify differential enriched transcription factor binding motifs found in these different gene lists.

Aim 3 – Confirmation of ERbeta target genes focusing on prostatic angiogenesis

Not initiated yet.

KEY RESEARCH ACCOMPLISHMENTS

- We have successfully generated C4-12-ERbeta, a cell line to be used as a model to study the genomic functions of ERbeta without the interference of ERalpha.
- We have mapped and conducted various comprehensive analyses of the ERbeta genomic binding regions upon 1 hour of estradiol induction using the C4-12-ERbeta cell line.
- We have discovered a crosstalk between ERbeta and EBF1 protein on a genomic scale.

REPORTABLE OUTCOMES

- We have generated a cell line, C4-12-ERbeta, as a good cell model to study the genomic function of ERbeta in an ERalpha-independent background.
- More importantly, we have reported here a novel crosstalk between ERbeta and EBF1, a major transcription factor in the process of B-cell differentiation. EBF1 was also reported to be downregulated in prostate cancer. The crosstalk between ERbeta and EBF1 is certainly an interesting topic in prostate cancer research.

CONCLUSION

In summary, using the C4-12-ERbeta cell line that we generated, we were able to map ERbeta genomic binding regions in an ERalpha-independent background. Our extensive analyses of these binding sites have revealed an interesting crosstalk between ERbeta and EBF1, a crucial transcription factor regulating the process of B-cell differentiation. Further experiments suggested a direct interaction between EBF1 and ERbeta, resulting in decreasing ERbeta protein stability and transcriptional activity in luciferase reporter assays. In order to assess the functionalities of ERbeta binding sites, we are applying the GRO-seq technology to detect nascent RNA being generated at time of ERbeta binding events. Detailed analyses, to be completed, will allow a better understanding of ERbeta genomic function as well as the role of EBF1 in this process. Ultimately, this will shed more lights on the role of ERbeta and EBF1 in prostate cancer development and progression.

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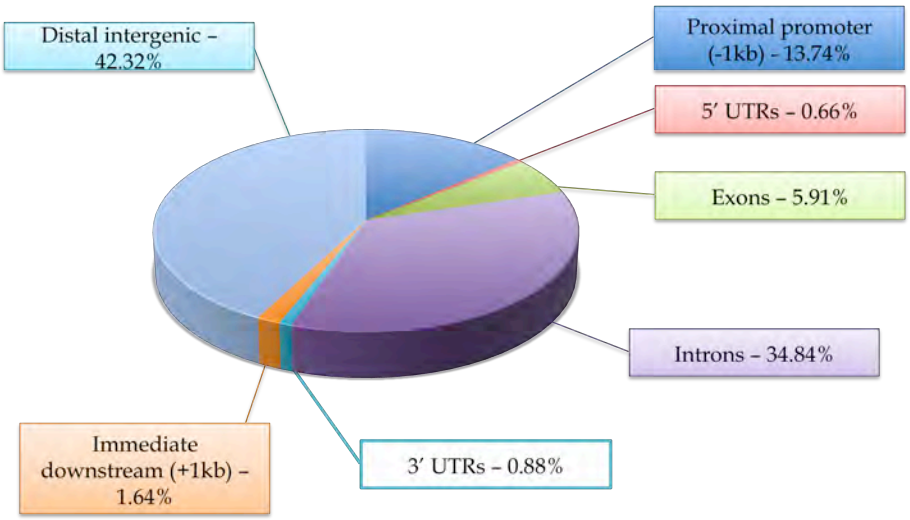
SUPPORTING DATA

A.



B.

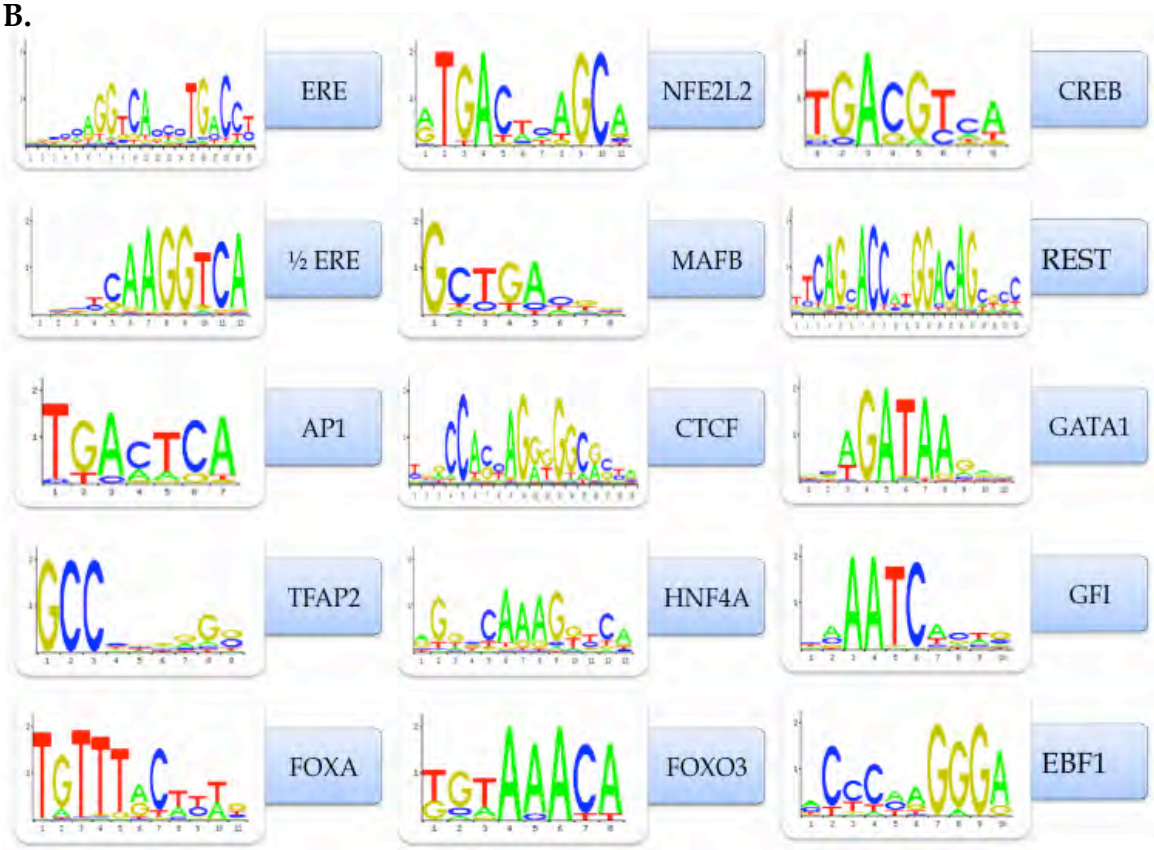


Figure 1 – Characterizations of ERbeta binding regions
A – Global distribution of 3166 ERbeta binding regions
B – Transcription factor binding motifs found enriched in ERbeta binding regions

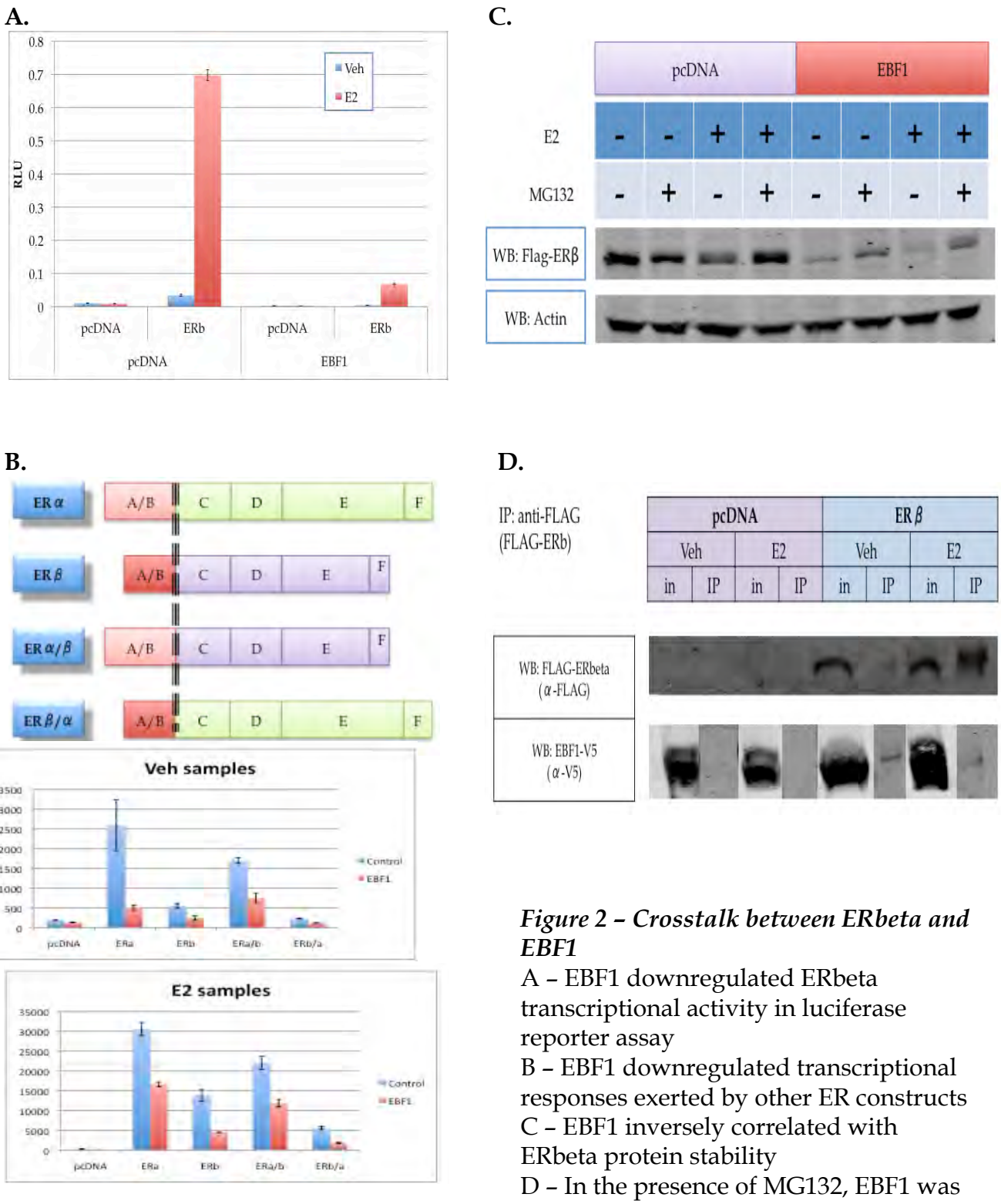


Figure 2 – Crosstalk between ERbeta and EBF1

A – EBF1 downregulated ERbeta transcriptional activity in luciferase reporter assay

B – EBF1 downregulated transcriptional responses exerted by other ER constructs

C – EBF1 inversely correlated with ERbeta protein stability

D – In the presence of MG132, EBF1 was coimmunoprecipiated with ERbeta

APPENDICES
N/A